Dihydroxyacetone Metabolism by Human Erythrocytes:
Demonstration of Triokinase Activity
and Its Characterization

By Ernest Beutler and Enriqueta Guinto

Dihydroxyacetone (DHA) was found rapidly to enter human erythrocytes. When incubated with intact red cells, $^{14}$C-labeled DHA rapidly appeared in intermediate metabolites and in lactate. Crude hemolysates were found to phosphorylate DHA and glyceraldehyde (GA) but not glycerol. Fifty-fold purification of the phosphorylating enzyme was achieved by removal of hemoglobin on CM-52 and Sephadex G-200 chromatography. During purification there was no change in the ratio of DHA to GA utilization. The enzyme had a pH optimum of 6.6, a $K_m$ of 0.5 $\mu$M for DHA, and a $K_m$ of 11 $\mu$M for GA. With GA as substrate, the $K_m$ for (ATP-Mg)$_2$ complex was 1.45 mM, and no cooperative interaction was observed. With DHA as a substrate, cooperative interaction with respect to (ATP-Mg)$_2$ complex was found with an $n$ value of 1.4 and a $K$ of 0.44 mM. At temperatures between 15° and 40°C, the enzyme activity gave a linear Arrhenius plot, with deviation from linearity at lower temperature levels. The activity of the enzyme was $0.151 \pm 0.011$ U/g Hb in five normal subjects, and only slightly increased levels were observed in a patient with marked reticulocytosis. The DHA-phosphorylating activity of erythrocytes is attributed to the activity of triokinase (ATP:D-glyceraldehyde-3-phosphotranserase; E.C.2.7.1.28). Although DHA is rapidly cleared from blood after injection into rabbits, substantial metabolism is possible for a prolonged time after administration because of the very low $K_m$ of the enzyme for DHA. The capacity of red cells to utilize DHA may prove to be useful in the maintenance of 2,3-diphosphoglycerate (2,3-DPG) levels during blood storage and in the in vivo manipulation of red cell 2,3-DPG levels.

When blood is stored in acid-citrate-dextrose (ACD) solution, rapid depletion of 2,3-diphosphoglycerate (2,3-DPG) results in a marked left shift of the oxygen dissociation curve. Various additives have been studied in an attempt to prevent or retard 2,3-DPG depletion. Recently, in surveying a large number of such agents, Brake and Deindoerfer discovered that the addition of dihydroxyacetone (DHA) to preservative mixtures enhanced the maintenance of 2,3-DPG levels.3

We now demonstrate that intact erythrocytes have the capacity to incorporate dihydroxyacetone into metabolic intermediates in the intact red cell and that, in the presence of ATP and Mg$^{2+}$, hemolysates have the capacity to phosphorylate DHA and glyceraldehyde (GA) to the normal glycolytic inter-
mediates, dihydroxyacetone phosphate (DHAP) and glyceraldehyde phosphate (GAP). These studies demonstrate, for the first time, that the enzyme that carries out such phosphorylation, triokinase, is present in hemolysates. We have partially purified and characterized this enzyme.

MATERIALS AND METHODS

Glycerol was obtained from J. T. Baker Chemical Co. Enzymes and other biochemical agents were obtained from Sigma Biochemical Corp., except for α-glycerophosphate dehydrogenase, which was obtained from Calbiochem.

Two methods of demonstrating the capacity of hemolysates to phosphorylate substrate were employed. A two-stage assay was used primarily in hemolysates and an assay linked directly to NADH oxidation through α-glycerophosphate dehydrogenase was used for studies of the kinetic properties of the enzyme. In each case, 1 U of enzyme is that amount that phosphorylates 1 μmole of substrate/min.

Two-Stage Assays

The following reaction mixture was used: Tris-HCl buffer, 1 M, pH 7.5 (1 M glycine, pH 9.5, for glycerol) 100 μl; 0.1 M MgCl₂, 100 μl; 20 mM ATP, 100 μl; 0.1 M dihydroxyacetone (DHA), glyceraldehyde (GA), or glycerol, 100 μl; hemolysate, 1:20, 500 μl; and water to a total volume of 1 ml.

The mixture was incubated at 37°C for 60 min, unless otherwise indicated, and the reaction was stopped by the addition of 2 ml of 4% perchloric acid. A blank system without substrate was included for each hemolysate. After centrifugation, 1.5 ml of the supernatant were neutralized with 1 M K₂CO₃, using methyl-orange as an indicator. The final volume was adjusted to 2 ml.

When crude enzyme preparations are assayed, phosphoric acid esters of DHA or of GA are interconverted to one another by the triosephosphate isomerase (TPI) in the preparation and to fructose diphosphate (FDP) by the aldolase in the preparation. For this reason, it is necessary to measure the sum of the dihydroxyacetone phosphate (DHAP), glyceraldehyde phosphate (GAP), and the fructose diphosphate (FDP) formed in the reaction mixture. This was done by adding 100–500 μl of the neutralized perchloric acid extract to a 1 ml cuvette together with 1 M Tris-HCl buffer, pH 7.5, 100 μl; 2 mM NADH, 100 μl; hemolysate, 1:20, 500 μl; and water to a total volume of 1 ml. The optical density of the mixture was measured at 340 nm before and after addition of 5 μl of TPI (2400 IU/ml), 5 μl of aldolase (100 IU/ml), and 5 μl of α-glycerophosphate dehydrogenase (580 IU/ml). The change in optical density was divided by 6.22 (the mM extinction coefficient of NADH) to give the number of μmoles of phosphorylated reaction product (GAP + DHAP + 2 × FDP) in the cuvette. The number of μmoles of product formed was calculated by subtracting the blank value from this value and correcting for dilution in the assay system and in preparation of the perchloric acid (PCA) extract. To estimate in the PCA extract the formation of α-glycerol phosphate when glycerol served as substrate, the following system with a final volume of 1.000 ml was used: 1 M Tris-HCl buffer, 7.5, 100 μl; 10 mM NAD, 100 μl; 200 mM hydrazine sulfate, 100 μl. The optical density was measured before and after addition of 5 μl α-glycerophosphate dehydrogenase (580 IU/ml).

Direct NADH-linked Assay System

The formation of glyceraldehyde phosphate (GAP) and of dihydroxyacetone phosphate (DHAP) was measured in a direct spectrophotometric assay using the following incubation system: 1 M Tris-HCl buffer, pH 8.0, 100 μl; 0.1 M MgCl₂, 50 μl; 50 mM ATP, 100 μl; 2 mM NADH, 100 μl; 58 U/ml α-glycerophosphate dehydrogenase, 50 μl; 2500 U/ml TPI, 5 μl (used with GA substrate only); hemolysate or enzyme, as indicated; and water to make a volume of 0.950 ml. Fifty microliters of a 20 mM solution of GA or DHA were added to the reaction system, and water was substituted for substrate in the blank. The decrease in optical density was measured at 340 nm at 37°C. A blank from which
hemolysate or enzyme preparation had been omitted was also determined and subtracted from all values.

Dihydroxyacetone concentrations were measured by noting the change in optical density at 340 nm after addition of 10.5 U of glycerokinase (Sigma) to a system containing: DHA solution, 2 mM ATP, 2 mM MgCl₂, 0.2 mM NADH, 3 U/ml α-glycerophosphate dehydrogenase, and 50 mM Bis-Tris, Tris, glycine buffer, pH 9.5.

Protein determinations were carried out by the method of Lowry et al.¹⁴

RESULTS

Utilization of Dihydroxyacetone by Intact Erythrocytes

Freshly drawn heparinized human blood was mixed with DHA to give a final concentration of approximately 50 mM by weight. The blood was centrifuged immediately in the cold; the plasma was removed from the packed cells, and perchloric acid extracts were made of both plasma and erythrocytes. The DHA concentration in the perchloric acid extract was measured. The concentration of DHA in the plasma was 46 mM and in the red cells was 32 mM. Assuming that the erythrocytes containing 70% water, this represents equal distribution between plasma and red cell water. The rapid penetration of dihydroxyacetone into erythrocyte was also confirmed by adding 0.1 ml of packed cells to a 2.5% aqueous dihydroxyacetone solution. The erythrocytes lysed immediately, indicating that the dihydroxyacetone rapidly equilibrated across the erythrocyte membrane.

¹⁴C-Dihydroxyacetone (International Chemical and Nuclear Corp.) was diluted in water and passed through a Dowex-1 formate column to remove radioactive impurities. Five microcuries of this preparation (containing 0.08 μmoles of DHA) and 5 μmoles of nonradioactive DHA were mixed and added to 80 ml of freshly drawn heparinized human blood. Forty milliliters were immediately deproteinized with 2 volumes of 10% (w/v) trichloroacetic acid (TCA). The precipitate was reextracted with 2 volumes of 5% TCA. The pooled TCA extracts were extracted repeatedly with diethyl ether, and their pH was adjusted to 6 with dilute ammonia. The remaining 40 ml of blood containing the ¹⁴C-dihydroxyacetone were incubated for 30 min under 5% CO₂-95% air and were then extracted with TCA in the same manner as the unincubated sample. The extracts were chromatographed on a 1.5 × 30 cm Dowex-1 formate column in a linear 0–5 N ammonium formate gradient, pH 3.0, as described by Bartlett.⁵ After 30-min incubation, approximately one-half of the radioactivity was found to be symmetrically associated with peaks containing lactate, monophosphoglycerate, and fructose diphosphate, and 2,3-DPG.

Demonstration of DHA- and GA-phosphorylating Activity in Crude Hemolysates

To determine whether red cell hemolysates possessed the capacity to phosphorylate DHA, GAP, or glycerol, 1 volume of packed washed erythrocytes was lysed in 9 volumes of EDTA-β-mercaptoethanol-NADP stabilizing solution,⁶ and the lysate was centrifuged at 5090 g at 4°C for 10 min.

Using the two-stage assay method, it was found that incubating these
crude hemolysates with either DHA or GA resulted in formation of phosphorylated derivatives. The rate of formation was linear with time for 90 min and linear with hemolysate concentration over a range of 0.1–0.5 ml of the 1:10 crude hemolysate.

Incubation with glycerol, in contrast, failed to result in the formation of any detectable glycerol phosphate.

Partial Purification

One hundred milliliters of 10 mM sodium citrate buffer, pH 6.0, were added to 25 ml of packed washed cells from which the buffy coat had been carefully aspirated. Stroma was removed by centrifugation at 12,100 g for 20 min. The supernatant hemolysate was dialyzed against the citrate buffer overnight, and approximately 200 g of Whatman CM 52, which had been equilibrated with the same buffer, were added. The hemoglobin was adsorbed to the CM 52. The supernatant and two washings of the ion exchange resin were pooled and concentrated against carbowax. The concentrate was chromatographed over Sephadex G-200 equilibrated with a pH 7.0, 10 mM Bis-Tris buffer. The peak tubes eluting at a position representing a molecular weight of approximately $1 \times 10^5$ represent approximately 50-fold purification of the enzyme, and this preparation was used for further studies. Greater purification was made difficult by the apparent instability of the enzyme to (NH$_4$)$_2$SO$_4$ fractionation and when absorbed to either DEAE- or ECTEOLA-cellulose columns.

Substrate Specificity of the Enzyme

Crude hemolysate showed the capacity to utilize DHA or GA at an approximately equal rate, using either of the two-step or direct NADH-linked assay. The rate remained the same at each of the two purification steps (Table 1), and the ratio of GA to DHA utilization was constant in all of the fractions eluted from Sephadex.

Like the hemolysate, the purified enzyme had no detectable capacity to phosphorylate glycerol.

Enzymatic Characteristics

The effect of pH on activity of the enzyme was studied by using a buffer containing 100 mM each of Bis-Tris, Tris, and glycine. The pH of this triple

<table>
<thead>
<tr>
<th>Table 1. Partial Purification of Triokinase</th>
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<tbody>
<tr>
<td>Fraction</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>(1) Crude hemolysate</td>
</tr>
<tr>
<td>(2) CM-52, carbowax-treated</td>
</tr>
<tr>
<td>(3) Sephadex G-200 peak fractions</td>
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</tbody>
</table>
Fig. 1. The pH activity curve of DHA-phosphorylating activity of partially purified red cell enzyme. ATP and magnesium concentrations were each 5 mM. DHA concentration was 1 mM. System was buffered with 50 mM each of Bis-Tris, and glycine. Abscissa is the cuvette pH at 37°C. The pH optimum curve is similar when GA serves as substrate, but the optimum is about 0.4 pH units higher.

Fig. 2. Effect of DHA concentration on DHA-phosphorylating activity of partially purified red cell enzyme. Measurements were made fluorometrically, in the system described in the text.

buffer was varied between 5.5 and 10.5 (as measured at 25°C) by adding sodium hydroxide or hydrochloric acid, and 500 µl of the buffer were used in each 1 ml assay system. The pH-activity curve was plotted according to the actual pH of the buffer when diluted in the assay system and warmed to 37°C. Under these conditions, the pH optimum of the enzyme was approximately 6.6 when DHA served as substrate (Fig. 1). With GA as substrate, the pH curve was very similar with a slightly higher optimum, pH 7.0.

The enzyme was shown to have a remarkably low \( K_m \) for dihydroxyacetone (Fig. 2). Indeed, it was not possible to measure its Michaelis constant spectrophotometrically, and measurements were, therefore, carried out photofluorometrically in a Turner fluorometer using a Corning 7-60 primary filter and Corning 3-72 secondary filter. The assay system contained in final concentration: 0.1 M Tris-HCl, pH 7.5; ATP, 5 mM; MgCl₂ 5 mM; \( \alpha \)-glycerophosphate dehydrogenase, 0.75 U/ml; NADH, 3 µM; and DHA concentrations varying
Mammalian tissues have been reported to contain two enzymes that have

![Fig. 3. Effect of GA concentration on phosphorylating activity of partially purified red cell enzyme. Assay conditions are given in the text.](image)

from 0.62 to 12.5 μM. The K_m of the enzyme for glyceraldehyde was 11 μM (Fig. 3).

The K_m of the enzyme for ATP-magnesium was studied at pH 7.5 in Tris buffer, maintaining equimolar concentrations of ATP and magnesium, varying from 0.2 to 5 mM. The results are plotted in terms of the ATP-magnesium complex, as suggested by Frandsen and Grunnet, calculating the ATP-magnesium complex concentration from a dissociation constant of $1.818 \times 10^{-8}$. When GA served as substrate, the relationship between $(ATP-Mg)^{2-}$ concentration and enzyme velocity followed classical Michaelis-Menten kinetics, and a single straight line was obtained on a Lineweaver-Burk plot. This was not the case when DHA was substrate. Under these circumstances there was evidence of cooperative interaction, and a straight line was not obtained on a Lineweaver-Burk plot. However, as shown in Fig. 4, an excellent straight-line fit was obtained using a Hill plot \[ \log S vs. \log \]

\[ \frac{v}{V_{max}} - v \]

The n value of 1.0 with GA as substrate and of 1.4 with DHA as substrate indicates that cooperative interaction was observed only with DHA. The K_m for $(ATP-Mg)^{2-}$ when GA was a substrate was 1.45 mM, while the K with DHA as substrate was 0.44 mM.

The effect of temperature on the enzyme assay is shown in Fig. 5. An Arrhenius plot (log velocity vs. reciprocal of absolute temperature) gave a straight line in the region between 15°C and 40°C, but there was consistently deviation from linearity at lower temperature levels, so that activity of the enzyme at 0°C was approximately 2% of that found at 37°C.

**Normal Values**

Erythrocytes from five normal subjects were assayed for capacity to phosphorylate DHA using the direct NAHD-linked system. The mean and standard error of the activity was $0.151 \pm 0.011$ U/g Hb. The red cells of a subject with a 10% reticulocyte count had an activity of 0.180 U/g Hb.

**DISCUSSION**

Mammalian tissues have been reported to contain two enzymes that have
the capacity to phosphorylate DHA. One of these enzymes is glycerol kinase (ATP:glycerol phosphotransferase; E.C.2.7.1.30); the other enzyme is triokinase (ATP:D-glyceraldehyde-3-phosphotransferase; E.C.2.7.1.28). The properties of these two enzymes are summarized in Table 2. One of their distinguishing characteristics, as shown in the table, is that triokinase has the capacity to phosphorylate GA and DHA, but not glycerol. In contrast, glycerokinase phosphorylates DHA and glycerol, but not GA. This characteristic of the two enzymes clearly indicates that the activity we have identified

Table 2. Comparison of the Effects of Glycerokinase and Triokinase

<table>
<thead>
<tr>
<th>Substrate specificity</th>
<th>Glycerokinase (E.C.2.7.1.30)</th>
<th>Triokinase (E.C.2.7.1.28)</th>
<th>Red Cell DHA-phosphorylating Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydroxyacetone</td>
<td>+9</td>
<td>+10</td>
<td>+</td>
</tr>
<tr>
<td>D-Glyceraldehyde</td>
<td>+9</td>
<td>+10</td>
<td>+</td>
</tr>
<tr>
<td>L-Glyceraldehyde</td>
<td>-9</td>
<td>0+</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+9</td>
<td>0+</td>
<td>0</td>
</tr>
<tr>
<td>Kinetic properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_m (DHA)</td>
<td>60 μM*</td>
<td>6 μM†</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>K_m (ATP)</td>
<td>28 μM†</td>
<td>20 μM§</td>
<td></td>
</tr>
<tr>
<td>K_m [ATP-Mg]2−</td>
<td>170 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH optimum</td>
<td>9.8**</td>
<td>7.0††</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*Pigeon liver.11  
†Rat liver, at 2.0 mM (ATP-Mg)2−.7  
‡Pigeon liver with glycerol for substrate.11  
§Beef liver.13  
||Rat liver.12  
‖Rat liver with GA for substrate.7  
**Rat liver with glycerol substrate.9  
††Beef liver.13  
‡‡Guinea pig liver.10,14
in hemolysates is most closely related to triokinase and does not represent
glycerokinase activity. The other characteristics of the human red cell hemo-
lysat activity also resembled much more closely the properties previously
described in rat liver for triokinase than those described for glycerokinase.

The identification of triokinase activity in human erythrocytes may prove to
be of practical importance. The activity of the enzyme in normal red cells,
0.15 U/g Hb, represents about 25% of the average maximal activity of
uninhibited hexokinase. While hexokinase is relatively strongly inhibited by
its product, glucose-6-P-and by 2,3-DPG, we have found that triokinase is
only slightly inhibited by high levels of its reaction product, dihydroxyacetone
phosphate, and by 2,3-DPG. Triokinase activity is, therefore, clearly suffi-
cient to play a significant role in erythrocyte metabolism. Triokinase can
phosphorylate glycoldehyde to glyceraldehyde phosphate and dihydroxy-
acetone to dihydroxyacetone phosphate. Both of these phosphate esters are
normal metabolic intermediates only a few steps before the formation of 2,3-
DPC. Glyceraldehyde is a highly reactive compound that, much like its ana-
logue, formaldehyde, fixes erythrocytes. It is, therefore, not likely to be useful
in red cell preservation. In preliminary studies, Brake and Deindoerfer
have found that 20 mM GA does not aid in 2,3-DPG preservation and that it
appears to produce red cell damage. In contrast, dihydroxyacetone may prove
to be a very useful substrate. Thus, when added to stored blood, it can act
as substrate for the maintenance of 2,3-DPG levels. Since the pH optimum
for its utilization is much lower than that of hexokinase, the formation of
lactic acid during storage is likely to have less of an effect on utilization of
this substrate than on that of glucose.

A remarkable feature of this enzyme is its extraordinarily low \(K_m\) for DHA.
Such a low \(K_m\) for a sugar-metabolizing enzyme must be very unusual. By
comparison, we have found the \(K_m\) of human red cell hexokinase to be 200
times this high. This implies that the red cell can utilize dihydroxyacetone
at concentrations only \(1/200\) of the concentrations of glucose. In preliminary
studies, we have found that DHA is very rapidly cleared from the blood of
rabbits following intravenous injections; the half time was approximately 3½ min. However, because of the low $K_m$ of red cells for this substrate, a single injection of a 9% (1 M) solution of DHA of 1% of the blood volume would give sufficiently high levels to metabolize for approximately 15 half-lives or almost 1 hr, if the initial clearance rate is maintained. If, as seems probable, the clearance rate slows as the level of dihydroxyacetone falls, a single rapid injection could provide metabolizable levels of substrate for a considerably longer period of time. Raising red cell 2,3-DPC levels by means of injection (or feeding) of DHA may make possible more efficient delivery of oxygen to tissues and could, therefore, be useful in shock, coronary artery disease, congestive heart failure, and other disorders. Such results can also be achieved by the infusion of inosine, pyruvate, and phosphate, but the large amount of uric acid formed from inosine will surely be an important limiting factor in the use of this combination. In contrast, DHA should be completely metabolized to lactate and is unlikely to have any toxic side effects.

Further studies will be required to assess the effect of DHA in blood preservation and in the treatment of hypoxic states. The present studies establish that red cells possess the enzymatic machinery for the utilization of this substrate.

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